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Polymorphism in the C-mos gene and productive traits in captive-bred scorpion mud turtles

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ABSTRACT. *Kinosternon scorpioides* is a freshwater turtle in need of conservation due to its vulnerability and population decline caused by predatory extraction from the natural environment and habitat destruction and degradation. Consequently, previous studies have focused on indicating the species' productive potential through productive features associations that aid the captive breeding system, but no one has explored candidate genes for these traits through molecular markers. Therefore, the C-mos gene was evaluated by the polymerase chain reaction technique (PCR) to look for polymorphisms useful in genetic analyses of a population raised in the Embrapa Eastern Amazon captivity, given its role in the maturation and regulation of oocyte transition and skeletal muscle development. The aim

was to look for these polymorphisms, characterize them, and link them with body weight (BW) and the average number of eggs (NE) traits. Thus, the DNA of 75 animals was extracted, sequenced, and the allelic and genotype frequency, observed and expected heterozygosity, inbreeding coefficients (FIS), and Hardy-Weinberg probability measures were assessed with GENEPOP software. The analysis of variance (ANOVA) one-way test at a 5% level of significance was used to check if BW and NE traits differ between different genotypes. The results revealed a non-synonymous polymorphism resulting in three genotypes: CC (49%), CT (42%), and TT (9%). However, these genotypes were not statistically associated with the body weight or number of eggs of the animals. Although this polymorphism is suitable to be used as a molecular marker in population genetic analysis involving species raised in captivity.

Key words: Capitivity; Captive breeding; Gene; *K. Scorpioides*; Polymorphism.

INTRODUCTION

The semi-aquatic turtle *Kinosternon scorpioides*, also known as muçuã, jururá and scorpion mud turtle, is a freshwater turtle in need of conservation due to its vulnerability and population decline caused by predatory extraction from the natural environment (Cristo et al., 2017) and habitat destruction and degradation (Berry and Iverson, 2011). It is importance for the Amazonian population stems from the demand for meat consumption, mainly by traditional people but also by people with better purchasing power (Baía Júnior et al., 2010, Alves et al., 2012, Frugoli et al., 2015, Marques, 2016, Cristo et al., 2017).

Due to this, previous studies have focused on indicating the species' productive potential through productive features associations that aid the captive breeding system (Iverson, 2010, Costa et al., 2015, 2017). These associations showed that the larger the animal, the more eggs it lays. This information is valuable for selection and improvement projects, yet it lacks a foundation in molecular biology. In this case, the average number of eggs and body weight are important measures of phenotypic traits in captive breeding. The first one is related to the ability to replace and maintain the bred population, and the second is related to the possibility of getting heavier animals.

In different animal production chains, molecular markers studies have been important to the selection of interest traits (Caratachea, 2007, Maciel et al., 2021, Zeng et al., 2021). Among these markers, single-nucleotide polymorphisms (SNPs) are the most popular in aquaculture species (Yue and Wang, 2017, Huang et al., 2020) and have been shown to be efficient in growth traits association analyses with the asian turtle P. sinensis (Zeng et al., 2021). Although the species K. scorpioides has been little explored in the molecular biology field since there are no studies with candidate genes for productive traits.

Given this potential, the C-mos gene emerges as a candidate for genotypic associations with egg production and body weight, as it encodes a serine-threonine kinase that catalyzes the phosphorylation of proteins by transferring phosphate groups from ATP to serine or threonine, regulating the chemical and structural changes of these proteins up to their transcriptional control (Guadagno and Ferrell Jr, 1998, Silva et al., 2009). This gene acts under stimuli in germinative and

somatic vertebrate cells, in the maturation and regulation of oocyte transition in the first case, as well as in the regulation of correct mitosis progression, myogenesis, and skeletal muscle development in the second case (Gebauer and Richter, 1997; Lenormand et al., 1997; Sagata, 1997; Godoy et al., 2015).

Therefore, this study aims to look for polymorphisms in the C-mos gene, characterize them genetically, and link them to the body weight and number of eggs traits of scorpion mud turtles that have been raised in captivity in the Amazon region.

MATERIAL AND METHODS

Animals, ethical and authoritative aspects

The studied animals came from the Embrapa Eastern Amazon Germplasm Bank in Salvaterra, Pará.

This bank's purpose is to conserve the genetic resources of animals in the Amazon and is authorized by the State Secretariat for the Environment and Sustainability (SEMAS) for "scientific breeding of wild life for research purposes" under operating license number 7310 terms.

The research was conducted under controlled conditions in accordance with animal welfare guidelines determined by the National Council for the Control of Animal Experimentation (CONCEA in its original portuguese acronym), and the projects was approved by the Internal Technical Committee under the Embrapa Seg Codes 22.1306023.00.00/01-2016 and 21159.002097/2022-85.

Biological material collection, and biological data

From 75 adult animals, 2 mL of blood was collected from the dorsal occipital sinus in vacuum tubes containing EDTA, which were kept at -20 °C up to laboratory procedures.

To proceed with the statistical analysis, the measured traits were the average number of eggs (NE) from 2012 to 2017, and adult body weight (BW) with the 0.001g electronic precision scale aid, Gehaka® brand, model BG 8000. Additionally, other growth traits such as carapace length (CL), carapace width (CW), plastron length (PL), plastron width (PW), and carapace height (HCP) of all animals were measured with a 300-mm manual caliper with 0.02 mm precision for the supplemental analysis.

Laboratory procedures

DNA extraction

Genomic DNA was extracted from animal blood by the phenol-chloroform method developed by Sambrook et al., (1989), and its quality was checked in a 1% agarose gel. The DNA was then quantified, and its purity was determined with a spectrophotometer (NanoDrop® ND-1000) by the absorption range 260A/280A measurement. The samples with rates equal to or higher than 1.8 were selected for the following steps.

Primer design and PCR

A primer pair was designed to amplify conserved regions from C-*mos* gene sequences available for the genus *Kinosternon* in the GenBank database (NCBI, the National Center for Biotechnology Information). The primers were developed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) to amplify 427 pb fragments at the same hybridization temperature.

The DNA samples were subjected to PCR using primers Forward 5' AGCAAGAACAGGTTGGCATC 3' and Reverse 5' GGGGACCTGGAGACACACTA 3'. The reactions were performed in a final volume of 20 µL, using the PCM Master Mix kit (Cellco Biotec), following the manufacturer's recommendations. The reaction conditions were: 1x of the master mix, 2 ng of DNA in concentrations between 50 - 80 ng/µL. The temperature and cycling conditions were: an initial denaturation step at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, hybridization at 61°C for 30 seconds, elongation at 72 °C for 45 seconds, and a final elongation step at 72°C for 3 minutes. All reactions were carried out in a CFX96 thermal cycler (Bio-Rad®). The reaction products were visualized on a 1.5% agarose gel.

DNA purification, sequencing, and analysis

The PCR products were purified using Ludwig Biotechnology DNA purification kit, followed by Sanger sequencing method with the Big Dye Kit, according to the manufacturer's recommendations. The sequences were determined by the automatic sequencing detector ABI 3500 Applied Biosystem.

Each sequence of electropherogram was analyzed first in FinchTV software, version 1.4.0 (https://digitalworldbiology.com/FinchTV) for SNP detection and sequence edition based on the species and other chelonian genus sequences deposited in the GenBank as references. Then, all sequences were aligned in the BioEdit 7.2 software (Hall, 1999).

Data statistical analysis

The population genetic analysis based on allele and genotype frequencies, the F_{IS} inbreeding coefficient, and the Hardy-Weinberg probability (HWE) was performed using the Genepop software, version 4.7.5 (Raymond and Rousset, 1995).

The Rstudio desktop software, version 4.2.2 (R Core Team, 2018), including the ggplot2 package, was used for statistical evaluation. The data normality was checked by the Shapiro-Wilk test as a premise for associations of the different genotypes with the variables body weight and average number of eggs, using an ANOVA one-way test at a 5% significance level.

Additionally, an exploratory analysis using descriptive statistics and graphical interpretation was conducted to refine the variables. With the same significance level, the Pearson Correlation test was performed to verify the associations among variables.

RESULTS

The primers successfully amplified the gene region studied, generating a 427pb fragment as a PCR product. Sequencing of this fragment revealed an SNP at the g.454 C>T position (Figure 1), based on the reference sequence of *Mauremys mutica* species, ID 123362854 deposited in Genbank. It is a

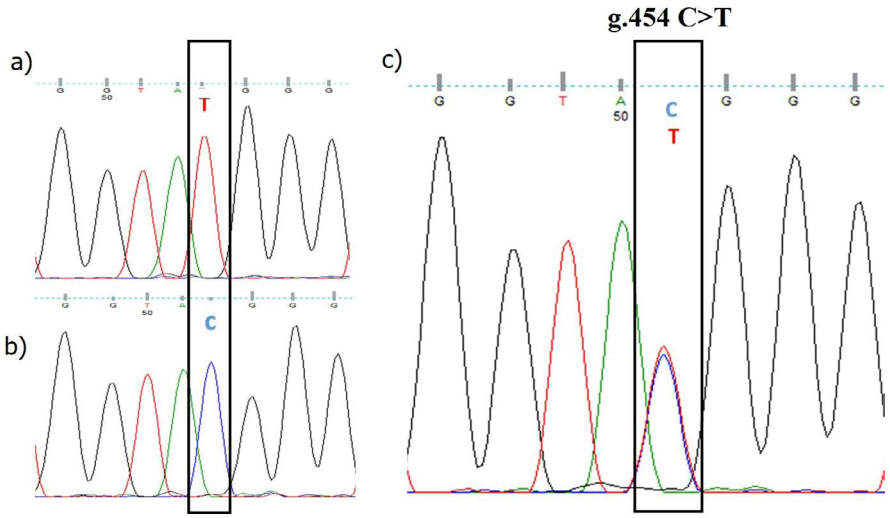

Figure 1. Electropherogram **sequences** displaying the non-synonymous polymorphism and the three genotypes found in the *K. scorpioides C-mos* gene. a) TT homozigote, b) CC homozigote and c) CT heterozigote.

Table 1. Genetic characterization of the *K. scorpioides C-mos* gene, Amazon.

Genotype	Allele	Ho_{ex}	Ho_{ob}	He_{ex}	He_{ob}	F_{IS}	HWP
CC (0,49)	C (0,7)	0,43	0,45	0,32	0,3	0,06	0,6
CT (0,42)							
TT (0,09)	T (0,3)						

Hoob = observed homozygosity; Ho_{ex} = expected homozygosity He_{ob} = observed heterozygosity; Heex = expected heterozygosity; F_{IS}, = inbreeding coefficient; HWP = Hardy-Weinberg probability. The genotypic and allelic frequencies are in parentheses. Total of 75 animals.

non-synonymous transition resulting from the amino acid exchange Arginine (R) with Tryptophan (W). Three distinct genotypes were identified: CC (homozygous for C alleles), TT (homozygous for T alleles), and CT (heterozygous). The number of genotypes, allele, expected and observed homozygosity and heterozygosity, inbreeding coefficient (F_{IS}), and the result of the Hardy-Weinberg probability test (HWP) are all in Table 1.

The Hardy-Weinberg equilibrium did not differ significantly (P > 0.05) from the expected probability in the studied group. The observed homozygosity values (0.45) were slightly higher than the expected values (0.43), which were different from the observed (0.3) and expected (0.32) heterozygosity values, all of which were below 0.5. The positive value of the F_{IS} inbreeding coefficient (0.6) points to the animals consanguinity. The most frequent genotype was CC (0.49), followed by CT (0.42), and the least frequent is TT (0.09). The C allele is the most prevalent in about 70% of the sample.

Figure 2 shows that there were no significant associations between genotypes and the NE a) and BW b) variables in any groups, as indicated by p values of 0.7218 and 0.2679, respectively, i.e. p>0.05. The boxplot analysis for the first variable does not indicate significant differences based on the median line, as 50% of the animals in all groups laid approximately 4 eggs. However, individuals of the CC genotype showed higher variation in the average number of eggs, where the minimum

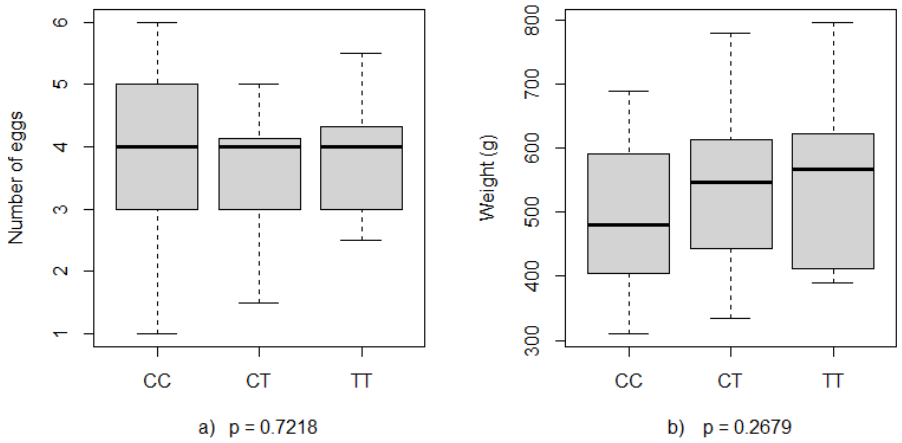

Figure 2. Number of eggs and weight (kg) of each genotype within the studied groups.

Table 2. Number of eggs and weight (g) performance of each genotype within the studied groups.

	Number of eggs		**Body Weight (g)**	
Genotype	Mean ± Sd	Min. - Max.	Mean ± Sd	Min - Max
CC	3.83 ± 1.33	1.0 - 6.0	493 ± 106	310 - 689
CT	3.58 ± 0.95	1.5 - 5.0	532 ± 117	335 - 778
TT	3.89 ± 1.05	2.5 - 5.5	549 ± 141	389 - 796

ANOVA one-way test at a 5% significance level. **Sd, standart deviation.**

was 1 and the maximum was 6 eggs per female. The TT group exhibited less variable in this regard, ranging between 2.5 - 5.5, i.e., the minimum number of eggs was higher in this group, confirmed by the mean value of 3.89 (Table 2). Similarly, the second variable, body weight, was slightly higher in individuals from the TT genotype, as 75% of all animals studied had at least 389 g, compared to 310g and 335g in the CC and CT groups, respectively. Animals that present the T allele have average weights higher than 500g and maximum weights of almost 800g.

DISCUSSION

This is the first scientific study to evaluate reproductive and growth traits in the scorpion mud turtle based on C-mos gene polymorphism. It was proven that the Embrapa population is diverse, which is important to avoid genetic erosion, what is common in captive-bred chelonians due to their long generation time and low fecundity (Williams and Osentoski, 2007). However, it was observed that the animals exhibited low heterozygosity, and the F_{IS} index leads to the understanding that the sample is under inbreeding pressure. This point was not deemed critical since Silva et al., (2011) have obtained 53 polymorphic fragments, indicating that species aren't in danger of genetic extinction since these two parameters of the genetic population (heterozygosity and the F_{IS} coeficient) are among the eight pointed out by Alacs et al., (2007) as important for the planning and execution of projects involving turtles.

This study investigated the application of SNP molecular markers to associate productive traits, as they have been efficient in studies with the genus and species *Kinosternon scorpioides*, given the evolution of molecular sequencing techniques (Saint et al., 1998; Iverson et al., 2013). These markers consist of variations in the DNA sequence that can guide genetic improvement initiatives regarding the seletion of individuals whose genotype favors interest traits (Caratachea, 2007); nevertheless, studies with the scorpion mud turtle are still limited to phylogenetic aspects (Caballero et al., 2022), confirming that the species needs to be explored in the field of molecular biology (Rodrigues et al., 2017).

However, the high cost of the technique continues to limit studies in this field, which could be minimized by making genomes available in public databases, but so far only 31 chelonians species, including subspecies, have this information in the genebank—that's not the case with the scorpion mud turtle.

A non-synonymous mutation at g.454C>T K. scorpioides sequence position produced three genotypes. However, statistical analysis did not find a significant link between these genotypes, BW and NE measurements. It is known that, unlike synonymous SNPs, non-synonymous SNPs alter the amino acid sequence of the resulting protein (Caratachea, 2007), but Bromberg and Rost (2007) explained that the obvious consequences of the exchange are not, necessarily, associated with these obvious functional or structural consequences, but these effects may manifest, when together with other polymorphic sites. This may be a possible explanation for the results here presented, as this conceptual issue does not seem rigid in chelonians since, in the opposite perspective, Zeng et al., (2021) transcriptome research has evidenced that the SNP g.9065462C>T, also resulting from a transition of the same base exchange in the IGF2R gene, is implicated in a significant association of the homozygous CC genotype with TT in slow-growing turtles of the species P. sinensis, even though it is a synonymous mutation.

In this research, the BW and NE traits were studied, since both are economically important to the chelonian production chain as well as for these animals conservation (Costa et al., 2017; Dantas-Filho et al., 2020; Andrade et al., 2021;Zeng et al., 2021). The body weight, for its nutrition-linked, which accounts for more than 50% of total turtle breeding costs (Andrade et al., 2021), and also because all other species growth traits have been strongly and positively correlated with this variable (Costa et al., 2015; 2017, view supplemental Figure). The average number of eggs because it is linked with the successful captive breeding system of chelonian that takes place with reproductive success (Williams and Osentoski 2007; He et al., 2010; Araújo et al., 2013; Trajano and Carneiro, 2019). In addition, previous studies have shown that the egg viability, around 20%, is still considered a critical point for Embrapa's captivity (Costa et al., 2017).

Thus, the studied animals vary in body weight and in the average number of eggs, even under the same environmental breeding conditions, according to the standard deviation values. Even though there were no statistical differences between these variables and the three genotypes found, an exploratory analysis of the data showed that the best performances were centered on the less common homozygous genotype (TT). For the reason that, in the most frequent group (CC), the maximum value of six eggs is due to only one female's performance. Regarding the body weight variable, the animals tend to be heavier, even if only one T allele is present. Zeng et al., (2021) explained this variation based on the differential gene expression of individuals with fast- and slow-growing phenotypes, proving that in chelonians this feature involves the complex signaling of more than 1,093 genes in adulthood, also influenced by gender. No different, the reproductive pathway of egg production in vertebrates is primarily controlled by the hypothalamic-pituitary-gonadal (HHG)

axis, with several genes involved in a complex chain of transcription factors, but knowledge of this axis's functioning in reptiles is still limited compared to that in mammals (Jones 2015; Bakshi et al., 2022).

Given this complexity, the gene here studied was chosen because it is an ortholog that encodes the Mos, a protein serine-threonine kinase i.e. is believed to be the main molecular mechanism controlling the meiotic and mitotic cell cycle of vertebrates through regulation and maturation-promoting factor (MPF) activation (Gebauer and Richter 1997; Sagata 1997; Oliveira et al., 2009). In female reproductive cells, the Mos, induced by progesterone, promotes maturation and regulation of oocytes growing within follicles (Gebauer and Richter 1997; Sagata 1997). The scorpion mud turtle vitellogenic follicle is characterized by a hypoechogenic round mass, which acquires an oval shape from 15.25mm onward and, with the advancement of maturation, becomes eggs suitable for laying around 28.8 mm (Chaves et al., 2012; Botega et al., 2016). In somatic cells, the constitutive expression Mos protein plays an important role in myogenesis and in the development or maintenance of specialized functions of skeletal muscle (Leibovitch et al., 1987; Lenormand et al., 1997), which is why (Godoy et al., 2015) indicate that the SNPs located in this gene should be better studied from animal captive breeding perspective.

CONCLUSION

The C-mos gene from the scorpion mud turtle bred-captive showed an SNP in the coding region. It is a non-synonymous polymorphism, and the C allele was the most frequent; however, the resulting genotypes were not associated with the body weight and number of eggs of the animals The SNP suggests that the population may be under selection pressure, as indicated by evidence of inbreeding, so this polymorphism can be used as a molecular marker in studies of population genetic analysis involving the species.

AUTHOR CONTRIBUTIONS

Ferreira, Hamoy, Marques, Pereira, and Silva Filho contributed to the conception, planning, methodology, development, and design of the study, as well as the writing of the manuscript. The latter two authors also supervised the research and were responsible for the formal analysis.

Silva, Marques, Silva, and Ferreira were involved in the provision of study materials, laboratory procedures, animal care, data estimation, and the compilation of all relevant documentation and research information.

All authors actively engaged in the discussion of results, provided insights, assisted in data interpretation, and contributed to the revision and finalization of the manuscript.

CONFLICT OF INTEREST

There is no conflict of interest in this study.

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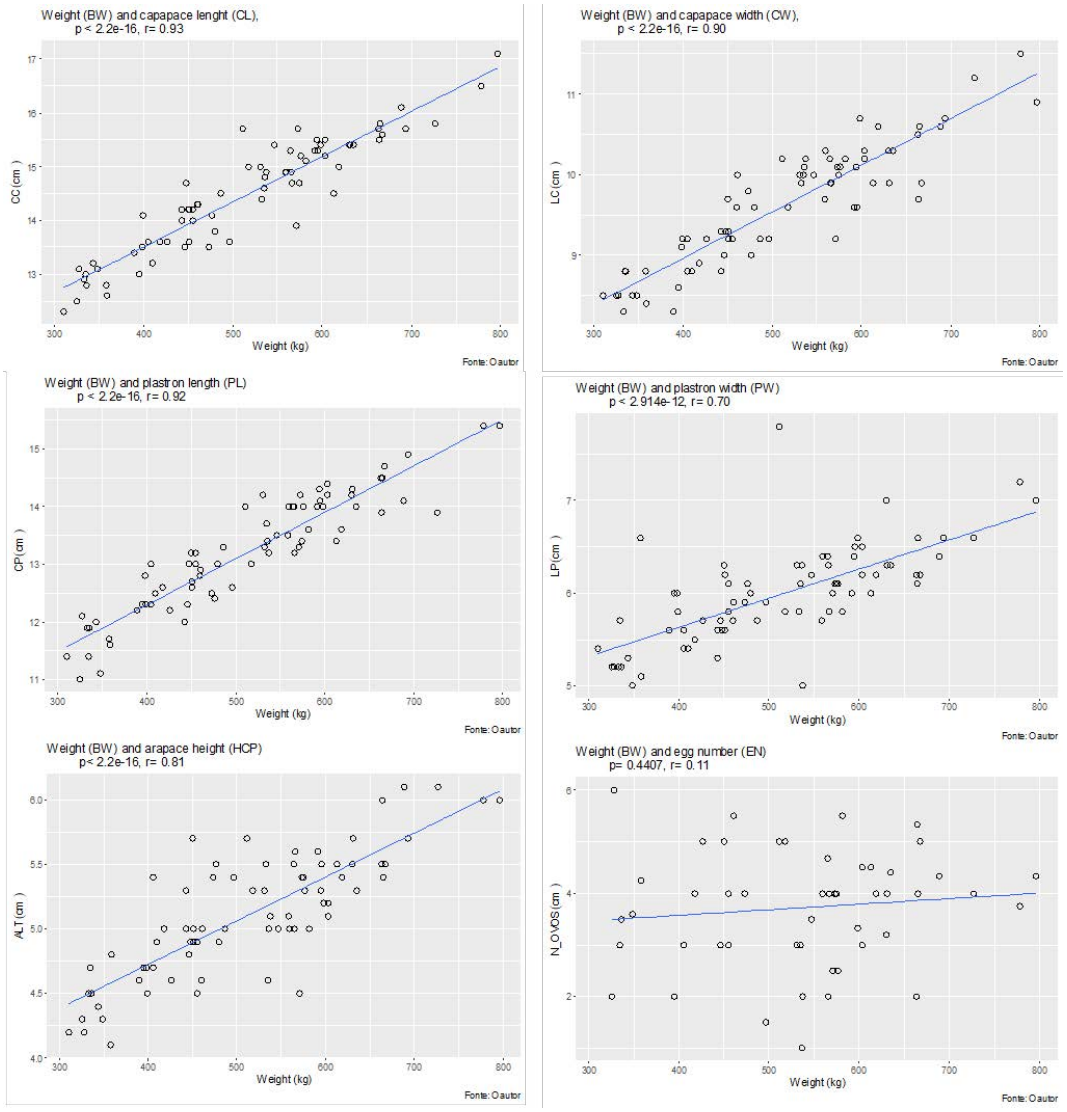

Supplemental Figure: Graphics showing the strong and positive Pearson's correlation between body weight (BW) and the other variables carapace length (CL), carapace width (CW), plastron length (PL), plastron width (PW), and carapace height (HCP). There is no correlation between BW and Number of eggs (NE).